

Local injections of tacrolimus-loaded hydrogel reduce systemic immunosuppression-related toxicity in vascularized composite allotransplantation

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Abbreviations:

ALT, alanine aminotransferase

AST, aspartate aminotransferase

BUN, blood urea nitrogen

CTL, cytotoxic T lymphocytes

DSA, donor-specific antibody

POD, postoperative day

TAC, tacrolimus

Teff, effector T cells

TGMS, triglycerol monostearate

TGMS-TAC, TAC-loaded TGMS hydrogel

Treg, regulatory T cells

VCA, vascularized composite allotransplantations

Abstract

Background: Routine application of vascularized composite allotransplantation (VCA) is hampered by immunosuppression-related health comorbidities. To mitigate these we developed an inflammation-responsive hydrogel for local immunosuppression. Here we report on its long-term effect on graft survival, immunological and toxicological impact.

Methods: Brown Norway-to-Lewis rat hind limb transplantations were treated either systemically with daily injections of 1 mg/kg tacrolimus or with subcutaneous intragraft injections of hydrogel containing 7 mg tacrolimus, every 70 days. Animals were monitored for rejection or other pathology for 280 days. Systemic and graft tacrolimus levels, regulatory T cells, and donor cell chimerism were measured periodically. At endpoint, markers for kidney, liver and metabolic state were compared to naïve age-matched rats.

Results: Both daily systemic tacrolimus and subcutaneous intragraft tacrolimus hydrogel at 70 day intervals were able to sustain graft survival for >280 days in 5 out of 6 recipients. In the hydrogel group, 1 graft progressed to grade 3 rejection at postoperative day (POD) 149. In systemic tacrolimus group, 1 animal was euthanized due to lymphoma on POD 275. Hydrogel treatment provided stable graft- and reduced systemic tacrolimus levels, and a 4 times smaller total tacrolimus dose compared with systemic immunosuppression. Hydrogel-treated animals showed preserved kidney function, absence of malignancies or opportunistic infections and increased hematopoietic chimerism compared to systemic immunosuppression.

Conclusions: Our findings demonstrate that localized immunosuppression with tacrolimus hydrogel is a long-term safe and reliable treatment. It may reduce the burden of systemic immunosuppression in VCA, potentially boosting the clinical application of this surgical intervention.

Introduction

Systemically administered tacrolimus (TAC) is the most commonly used immunosuppressant in vascularized composite allotransplantation (VCA)¹. However, of the 66 registered in the International Registry on Hand and Composite Tissue Transplantation hand transplant recipients, 26% suffered from elevated creatinine values, 32.3% – from opportunistic bacterial infections and 3 of them developed malignancies². These TAC-mediated morbidities are a barrier to the broader adoption of VCA. Transitioning patients from TAC to other immunosuppressants has been attempted, but with limited success². Consequently, the field has turned to “increasingly bold approaches in modifying immunosuppression”³, that need solid and conclusive preclinical data, demonstrating their feasibility, efficacy and safety.

Our group developed a graft-targeted inflammation-responsive hydrogel⁴⁻⁶ delivering TAC “on demand” – only when needed, with the aim to provide an effective future alternative or addition to systemic immunosuppression for patients. The hydrogelator – triglycerol monostearate (TGMS) – is biocompatible, biodegradable, generally recognized as safe by the US Food and Drug Administration, and can be loaded with therapeutically relevant amounts of TAC. TAC-loaded TGMS hydrogel (TGMS-TAC) releases TAC in response to inflammatory stimuli, and prolongs VCA survival with a single injection⁷.

Here we hypothesized that repeated subcutaneous intragraft injections of TGMS-TAC maintain long-term graft survival in the Brown Norway-to-Lewis rat hind limb allotransplantation model. We expected that TGMS-TAC-treated animals would have higher TAC concentrations in the graft and lower in the blood compared to daily systemic TAC treatment (standard of care), which should result in reduced off-target effects and

nephrotoxicity. Furthermore, we were interested in whether TGMS-TAC influences the dynamics of effector T cells (Teff), regulatory T cells (Treg), and chimerism.

Materials and Methods:

Male Brown Norway and Lewis rats (6-8 weeks old weighing 200 to 250 g) were purchased from Charles Rivers Breeding Laboratories, Germany. Animals were kept in specific pathogen-free conditions. Experiments were planned and carried out in agreement with current 3R and ARRIVE guidelines and approved according to Swiss animal protection laws by the Veterinary Authorities of the Canton Bern, Switzerland, approval no. BE94/15.

Brown Norway-to-Lewis rat hind limb transplantations were performed and animals were treated either with 1 mg/kg/day TAC systemically in the neck fold or every 70 days with 1 ml TGMS-TAC containing 7 mg tacrolimus (n=6 for each group). In the TGMS-TAC group, 4 subcutaneous TGMS-TAC depots of 250 μ L each were injected in the zones of biceps femoris, gastrocnemius, tibialis anterior, and vastus muscles, taking great care to distribute the amount of drug as evenly as possible intra and interindividually. The reinjection time point was chosen based on a pilot study showing that transplanted animals (n=5) treated with a single intragraft injection of 1 ml TGMS-TAC loaded with 7 mg TAC on postoperative day 1 (POD 1) rejected their graft on POD 83.4 ± 6.7 . Reinjection time point was defined as 14 days before rejection and set to POD 70. Graft rejection was evaluated macroscopically and graded as 0 = no rejection, 1 = erythema and edema, 2 = epidermolysis and exudation, and 3 = desquamation, necrosis, and mummification.

Rats were euthanized either once grade 3 rejection was reached or on day 280 (endpoint). Necropsy for immunosuppression-related side effects was performed. Kidney, as well as graft skin and muscle histology were evaluated by a blinded pathologist (Hematoxylin and Eosin

and/or Periodic acid–Schiff staining, as necessary). Kidney samples were graded according to the semiquantitative calcineurin inhibitor toxicity score by Kambham et al⁸. Histological grading of skin rejection was according to Banff classification⁹. Additionally skin and muscle lymphocyte infiltration, vasculopathy and necrosis were graded as 0 – none, 1 – minimal, 2 – moderate, 3 – extensive. Immunofluorescence analyses of IgG, IgM, C3b/c, C4b/c, C5b-9, CD45RA in graft skin and muscle were performed. Blood urea nitrogen (BUN) and creatinine; cholesterol and triglycerides; aspartate aminotransferase (AST) and alanine aminotransferase (ALT) as kidney, metabolism and liver markers, respectively, complete blood count, and donor-specific antibody formation were assessed in blood at endpoint.

Throughout the study TAC levels were measured in blood and skin biopsies retrieved from grafts and contralateral limbs at selected time points by LC-MS/MS.

Flow cytometry for obtaining Treg and chimerism levels was performed in blood at selected time points, and in graft and contralateral limb skin at endpoint.

Statistical analyses were executed with Prism software (GraphPad Software Inc., La Jolla, CA, United States). Statistically significant data were presented as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$. Statistical tests are specifically indicated under each figure.

Detailed materials and methods are available in SDC, Materials and Methods, in the online version of this article (SDC, <http://links.lww.com/TP/B584>).

Results

Periodic TGMS-TAC injections promote long-term VCA survival

To test if 1 ml TGMS-TAC loaded with 7 mg TAC reinjected every 70 days promotes long-term graft survival in a rat hind limb transplantation model, we compared TGMS-TAC

treatment to daily systemic immunosuppression using TAC at 1 mg/kg/day (Figure 1a). Five out of 6 animals survived until endpoint in each group (Figure 1b).

In the TGMS-TAC group, 1 animal was sacrificed due to grade 3 rejection at POD 149 (Animal 1, Figure 1c). One animal experienced 3 rejection episodes (Animal 2, Figure 1c). One animal started rejecting at POD 262, which reached grade 2 at endpoint (Animal 3, Figure 1c). One animal experienced mild rejection, which resolved temporarily and later reoccurred (Animal 4, Figure 1c). Two animals had no rejection episodes (Animals 5 and 6, Figure 1c). No local complications due to TGMS-TAC-injections were observed in any of the rats. In systemic TAC group, 1 animal was euthanized due to lymphoma on POD 275. Systemic immunosuppression was sufficient to maintain the grafts rejection-free throughout the duration of the treatment (Animals 7-12, Figure 1c).

Histopathological evaluation of graft skin at endpoint (POD 280) revealed no necrosis in any of the animals (Animals 1-12, Figure 1d). Two TGMS-TAC-treated animals were classified as Banff grade 3 (corresponding to macroscopic grade 2), and 1 as Banff grade 2 (corresponding to macroscopic grade 1) skin rejection (Animals 2-4, Figure 1d). One systemically treated animal was classified as Banff grade 2 skin rejection, although no macroscopic lesions were detectable (Animal 12, Figure 1d). No significant differences were found between the 2 groups in terms of lymphocyte infiltration ($P=0.2415$), vasculopathy ($P=0.1411$) or Banff grade ($P=0.1660$, Mann-Whitney test, Figure 1d). All observed rejection episodes were restricted to graft skin, with no signs of rejection in graft muscle (Figure S1, SDC, <http://links.lww.com/TP/B584>). However, TGMS-TAC-treated animals had significantly more pronounced atrophy of the muscle fibers as compared to systemic treatment ($P=0.0079$, Mann-Whitney test, Figure S1, SDC, <http://links.lww.com/TP/B584>).

As evident from Figure 1c, 2 rejection episodes in animal 2 markedly improved after TGMS-TAC reinjection. The first one – macroscopic grade 1 at the time of reinjection – was completely reverted within a week. The second one – macroscopic grade 2 – required 2 to 3 weeks to reduce to grade 1 (Figure 2) and within a month a complete recovery was observed, which, however, lasted only 3 weeks.

Reduced systemic but relevant tissue TAC levels with TGMS-TAC

To understand TAC distribution over time, TAC concentrations were measured in blood (biweekly), and in graft and contralateral limb skin (monthly). In blood, a burst release of TAC was detected in the first 72 h after the first TGMS-TAC injection. This peak was beyond the upper quantification limit of the LC-MS/MS analysis (ie, 65 ng/ml) during the first 24-48 h, afterwards it normalized to therapeutic levels (5 – 20 ng/ml). At POD 46 most animals had subtherapeutic levels (<5 ng/ml). Compared to the first TGMS-TAC injection, the following injections induced markedly weaker burst releases, followed by similar TAC release kinetics ($P<0.0001$ for first peak versus second, $P<0.0001$ for first peak versus third, and $P=0.0009$ for second peak vs. third, standard one-way ANOVA, Figure 3a). Trough levels of systemically treated animals were within the therapeutic range throughout the duration of the experiments (Figure 3a). Biweekly average TAC levels in blood of TGMS-TAC and animals showed that TGMS-TAC-treated animals had significantly lower TAC blood levels compared with systemically treated animals (9.29 ± 5.89 vs. 13.44 ± 4.44 ng/ml, respectively, $P=0.0060$, Mann-Whitney test, Figure 3b).

In TGMS-TAC-treated animals there was a nonsignificant trend towards higher TAC concentrations in the graft as compared to the contralateral limb skin during the first week,

corresponding to the burst release observed in blood ($P=0.1532$, paired t-test, Figure 4a). For the rest of the measured time points there were no statistically significant differences between TAC levels in graft and contralateral limb skin. In systemically treated animals there were no statistically significant differences between the TAC levels in graft and contralateral limb skin at all time points (Figure 4b). Monthly average skin TAC levels in TGMS-TAC graft skin were higher than in the respective contralateral limb skin (1.1 ± 1.49 ng/mg vs. 0.25 ± 0.19 , respectively, $P=0.0195$, Figure 4c). Under systemic treatment there was no significant difference between graft and contralateral limb skin (0.46 ± 0.39 ng/mg vs. 0.29 ± 0.16 ng/mg, respectively, $P=0.1094$, Wilcoxon test, Figure 4c). When compared between groups, TAC levels in both graft and contralateral limb skin were comparable ($P=0.7785$ and $P=0.4755$ respectively, Mann-Whitney test, Figure 4c).

Low antidonor antibody and complement activation

The formation of donor-specific antibody (DSA) was assessed by incubating donor thymocytes with plasma of transplanted animals and subsequent analysis by flow cytometry. There was no significant intergroup difference for binding of IgG to donor thymocytes, whereas IgM was significantly lower in the TGMS-TAC group at POD 280 as compared to systemic treatment (mean fluorescence – 75.56 ± 18.62 and 143.8 ± 41.09 respectively, $P=0.013$, Student's t-test, Figure 5a). At endpoint no significant antibody (IgG and IgM, Figure 5b) and complement (C4b/c, C5b-9, Figure S2, SDC, <http://links.lww.com/TP/B584>) deposition, or B cell infiltration was observed (CD45R, Figure S3, SDC, SDC, <http://links.lww.com/TP/B584>), except for increased C3b/c deposition under systemic treatment (integrated density 1018 ± 155.6 and 1500 ± 300.1 for TGMS-TAC and systemic

treatment respectively, $P=0.029$, Student's t-test, Figure S2, SDC, <http://links.lww.com/TP/B584>).

TGMS-TAC mitigates immunosuppression-related side effects

BUN in TGMS-TAC animals was higher than in naïve age-matched animals and lower than in systemically treated animals – 12.62 ± 1.6 , 5.9 ± 0.2 and 20.86 ± 4.86 mmol/l, respectively ($P=0.0023$ TGMS-TAC vs. systemic treatment, $P=0.0095$ TGMS-TAC vs. naïve, and $P<0.0001$ systemic treatment vs. naïve, Figure 6a). Creatinine was lower in TGMS-TAC and naïve age-matched animals compared to systemic treatment (27.2 ± 4.21 , 23.2 ± 2.68 , and 49.8 ± 17.08 μ mol/l, respectively, $P=0.0117$ TGMS-TAC vs. systemic treatment, $P=0.0039$ systemic treatment vs. naïve, Figure 6b). Histological analysis of kidneys revealed only minimal damage under both treatments (Figure S4, SDC, <http://links.lww.com/TP/B584>).

Cholesterol was comparable between groups (2.48 ± 0.39 , 2.35 ± 0.48 , and 2.88 ± 0.47 mmol/l for TGMS-TAC, systemic treatment, and naïve Lewis rats, respectively, Figure 6c). Triglycerides were similar in TGMS-TAC and naïve rats and decreased in systemic treatment group as compared to naïve rats (1.22 ± 0.54 mmol/l, 0.44 ± 0.29 mmol/l, and 1.75 ± 0.99 mmol/l for TGMS-TAC, systemic treatment and naïve Lewis rats respectively, $P=0.0236$ between naïve rats and systemic treatment, Figure 6d).

Hepatic enzymes were not significantly different between naïve rats and the 2 treatment groups. AST was 140.6 ± 97.18 U/L, 109 ± 52.72 U/L, and 80 ± 15.64 U/L for TGMS-TAC, systemic treatment, and naïve rats respectively, Figure 6e. ALT was 73.4 ± 29.57 U/L, 48.4 ± 29 U/L, and 50.4 ± 7.8 U/L for TGMS-TAC, systemic treatment, and naïve rats respectively, Figure 6f).

The complete blood count of TGMS-TAC and systemically treated animals at endpoint was comparable to naïve age-matched rats, except for total hemoglobin, mean corpuscular hemoglobin, platelet distribution width, and median platelet volume, which were lower under systemic treatment, compared either to naïve animals or to both naïve and TGMS-TAC-treated animals (one-way ANOVA, Table 1).

As mentioned above, under systemic treatment 1 of the 6 animals was euthanized at POD 275, due to markedly enlarged ipsilateral inguinal lymph node, accompanied by elevated white blood cell count (75.1×10^3 cells/ μ l) and apathetic behavior indicating pain and/or suffering. Histopathological analyses of the lymph node revealed aggressive lymphoma, most consistent with diffuse large B cell lymphoma (Figure 6g). Another animal from the same group had an increasingly firm and growing solid mass circumventing the graft, accompanied by a slow but steady increase of the white blood cell count until endpoint. Necropsy revealed a large encapsulated granuloma-like formation filled with granulated yellow-green substance. Histopathological analysis confirmed that the formation was an infected pseudo-cyst (Figure 6h). PCR analyses of its content revealed *Staphylococcus aureus* and *Proteus mirabilis*, commensal skin bacteria. In the TGMS-TAC group neither malignant nor infectious complications were observed.

TGMS-TAC therapy favors hematopoietic chimerism

To understand the dynamics of Teff and Treg, and chimerism under both treatments we analyzed blood at selected time points throughout the study. The gating strategy for Teff and Treg enumeration is shown in Figure S5 (SDC, <http://links.lww.com/TP/B584>), and for chimerism – in Figure S6 (SDC, <http://links.lww.com/TP/B584>).

Both treatment groups had significantly decreased amounts of circulating T cells compared to naïve rats. Initially there were significantly more T cells in the TGMS-TAC group than in systemically treated group (for example in postoperative week 2: 2087 ± 427 T cells/ μ l vs. 1163 ± 359 T cells/ μ l respectively – $P=0.0074$, Student's t-test). After 17 weeks of gradual decrease the difference of T cell counts between the TGMS-TAC and systemically treated group were no longer statistically significant (for example in postoperative week 19: 1825 ± 767 T cells/ μ l vs. 1290 ± 336 T cells/ μ l, $P=0.1486$, Student's t-test, Figure 7a). Three T cell populations – cytotoxic T lymphocytes (CTL), T helper cells and Treg – were separately analyzed, with additional focus on Helios⁺ and Helios⁻ Treg populations. The T helper cells were the most abundant T cell population and followed the total T cell dynamics (Figure 7b). There were no major differences in the CTL or the Treg populations between the 2 treatment groups over time (Figure 7c-f, respectively).

In terms of chimerism, in the first 11 weeks there was a significantly higher amount of circulating donor-derived cells in the TGMS-TAC group (Figure 8a). Donor-derived B cells, T helper cells, CTL, and monocytes were all significantly increased in the TGMS-TAC-treated group compared to systemic treatment for up to 23 weeks (Figure 8b-e). Circulating donor-derived granulocytes were initially high in both treatment groups (for example in postoperative week 2: 367 ± 92 and 314 ± 113 donor-derived granulocytes/ μ l for TGMS-TAC and systemic treatment, respectively). This number dropped to 58 ± 52 and 90 ± 36 cells/ μ l, respectively, at postoperative week 10 and remained low until termination of the experiment (Figure 8f). At endpoint peripheral blood monocytes isolated from graft and contralateral limb skin of both groups were analyzed using the same flow cytometry protocol. The cell count

was low and revealed no significant differences between the 2 treatment groups (Figure S7, SDC, <http://links.lww.com/TP/B584>).

Discussion

Our data show that repeated intragraft injections of TGMS-TAC sustain long-term graft survival with better toxicological and immunological outcomes as compared to systemic TAC delivery. Markers of kidney function (ie, BUN and creatinine) and complete blood analysis at endpoint, showed preserved kidney and hematological parameters of TGMS-TAC-treated rats as compared to systemic treatment. Unlike humans¹⁰, rat models require sodium depletion in order to develop significant TAC-induced kidney damage¹¹⁻¹³. Therefore, we speculate that the toxic effects reported in this study may be underrepresented, and that the TGMS-TAC treatment may potentially have more visible benefits in humans, especially in the kidney on a histological level.

Our study was also of sufficient duration to reveal possible complications of long-term immunosuppression. One systemically treated animal developed an infected pseudo-cyst containing commensal skin bacteria. Another developed an aggressive lymphoma. Lymphomas can arise spontaneously in ageing Lewis rats; however, their incidence during the first year of life of a male Lewis rat is extremely low¹⁴, suggesting that systemic immunosuppression contributed to its development. Necropsy of TGMS-TAC-treated animals did not reveal any malignancy or opportunistic infection, suggesting that localized immunosuppression could mitigate immunosuppression-related complications.

Local complications related to TGMS-TAC treatment, such as rash, alopecia, discoloration, atrophy or thinning of skin, or extracutaneous hydrogel extrusions were not observed.

Animals did not extensively groom or scratch the limb after injection indicating absence of local irritation. Stool was firm and urine was clear, suggesting no acute gastro-intestinal or renal complications resulting from TGMS-TAC either.

While providing better recipient outcomes, TGMS-TAC treatment resulted in inferior graft outcomes as compared to systemic treatment. Four of the 6 TGMS-TAC treated animals experienced at least 1 rejection episode. Rejecting TGMS-TAC treated animals had comparable systemic TAC levels to the nonrejecting TGMS-TAC treated animals. Moreover, we⁷ and others¹⁵ have demonstrated that localized immunosuppression promotes extended rejection-free graft survival in the setting of subtherapeutic systemic TAC levels. Therefore, we hypothesize that these rejections are not due to subtherapeutic systemic TAC levels, but rather to low intragraft TAC levels. According to Capron et al, tissue levels of immunosuppression provide a more accurate insight into actual efficiency of immunosuppression¹⁶. Reinjecting TGMS-TAC guided by local TAC levels, instead of fixed time points, could mitigate the observed rejections. To test this hypothesis, we plan to conduct TGMS-TAC studies in a porcine VCA model. In addition to being more clinically relevant, pigs provide the opportunity to collect frequent biopsies, sufficient to identify minimal threshold for intragraft TAC levels.

TGMS-TAC treated animals demonstrated increased muscle atrophy as compared to systemically treated animals. Calcineurin is involved in skeletal muscle hypertrophy and tacrolimus counteracts this effect¹⁷. However, to our knowledge, there have been no studies demonstrating that tacrolimus monotherapy causes direct myotoxicity, as conversely reported for tacrolimus in conjunction with statins¹⁸. Moreover, clinical cases of tacrolimus overdose have not reported effects on skeletal musculature¹⁹, suggesting that muscle atrophy is not

TAC related. Mechanical pressure of the hydrogel deposits on graft vessels or nerves resulting in muscle atrophy is not a likely explanation either, since grafts were all well-perfused and all animals used their limbs for walking until endpoint in both groups. The hydrogel itself has been previously described to be safe, biocompatible and biodegradable⁷. However, our data cannot rule out the possibility that muscle atrophy may be a hydrogel-related side effect, which could not develop in studies of shorter duration. Importantly, muscle atrophy is a known manifestation of chronic rejection, and we believe that this is the most likely explanation for our observations. Indeed, multiple acute rejection episodes have been correlated to chronic rejection, particularly in rat²⁰. However, nonrejecting TGMS-TAC treated animals also had high muscle atrophy scores, keeping the question of muscle atrophy a matter requiring further investigation.

A third and potentially problematic aspect of TGMS-TAC hydrogel could be the TAC burst release following TGMS-TAC injection. TGMS-TAC injections led to peaks in TAC blood levels that, with each subsequent injection, became significantly lower. Due to the enzyme responsiveness of the hydrogel, the most likely reason for the very high first peak is the elevated levels of inflammation-related enzymes resulting from the surgical trauma and ischemia-reperfusion injury. However, in our view, the burst release has arguably a negative impact, as high intragraft perioperative TAC levels were shown to prolong graft survival in the same experimental model²¹.

Each TGMS-TAC injection contained 7 mg of TAC and the total amount of TAC given over 280 days to TGMS-TAC-treated animals was 28 mg. In contrast, animals treated systemically with 1 mg TAC/kg/day received a total of 84 to 112 mg of TAC, depending on the weight of the rats, which ranged from 300 to 400 g. Consequently the systemic TAC levels in TGMS-

TAC treated animals were significantly lower than the trough TAC levels in systemically treated animals. Nevertheless, TAC levels in skin were comparable between the 2 groups. Interestingly, while in nonrejecting TGMS-TAC treated animals TAC levels were similar in transplanted and contralateral limb skin, upon rejection levels increased in the transplanted limb as compared to nonrejecting grafts and contralateral limb skin (Figure S8, SDC, <http://links.lww.com/TP/B584>). This supports the idea that rejection triggers the local release of the drug. We also found that at endpoint systemically treated animals had significantly decreased tissue TAC levels ($P<0.05$ in graft and $P<0.001$ in contralateral limb skin as compared to previous time point by paired t-test). We do not have an explanation for this observation, as reduced systemic TAC levels, or any observable physiological changes, did not accompany it.

In terms of immunological outcomes, the amount of circulating and intra-graft Treg was comparable between TGMS-TAC and systemic treatment. However, TGMS-TAC treatment was associated with higher and more persistent hematopoietic chimerism compared to systemic treatment. Chimerism is a protolerogenic factor²² and boosting it without aggressive preconditioning or bone marrow transplantation may be an attractive option to control anti-graft immunity in VCA. Despite elevated chimerism, most TGMS-TAC-treated animals experienced rejection episodes. Chimerism alone is not sufficient to prevent rejection and requires the support of higher Treg counts²³, which was not the case under both treatments. Moreover, it has been shown that robust chimerism cannot prevent rejection once immunosuppression is tapered in a porcine VCA model²⁴. The levels of chimerism in TGMS-TAC-treated animals, despite being elevated compared to systemic treatment, were still below the threshold required for tolerance²⁵. Therefore rejection due to reaching low intra-graft TAC

levels was not preventable by the achieved increase in chimerism with TGMS-TAC. Nevertheless, the possibility to use localized immunosuppression to increase chimerism levels to the “tolerogenic threshold”²⁵ represents an interesting opportunity that deserves further investigation.

In 2014 for the first time the VCA society dealt with antibody-mediated rejection in a presensitized face recipient, raising patient sensitization as the next frontier in the field²⁶. Recent studies in rat VCA model have clearly demonstrated that sensitized recipients experience accelerated rejection of both cell- and antibody-mediated nature²⁷. In our study we have not included a presensitized group, neither did our animals develop *de novo* DSA. Complement deposition²⁸, and tertiary lymphoid structure formation²⁹, which were also described as participants in the VCA rejection process, were also not detected, consistent with previous studies³⁰. Future studies addressing the efficacy of TGMS-TAC in a sensitized animal model would provide a strong argument on the potential and limitations of this therapeutic modality.

In view of clinical application, a combination of “the best of both worlds” – combining use of reduced systemic immunosuppression and local ‘on demand’ immunosuppression – might be envisaged to balance the outcomes of graft and recipient. Moreover, single-drug immunotherapies are not successful in clinical VCA. Multidrug immunosuppressive protocols are currently used in transplanted patients to guarantee an effective level of immunosuppression. Therefore, we believe that protocols involving localized immunosuppression in humans should further evolve by including multiple drugs to better control graft rejection.

In summary, this study demonstrates that the use of an enzyme-responsive drug delivery system for localized immunosuppression in VCA results in long-term graft survival with reduced drug-related side effects. These findings support the safety of this therapeutic possibility, and suggest a potential to mitigate immunosuppression-related morbidities in patients.

ACCEPTED

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Figure legends:

Figure 1 Long-term graft survival and graft histology are comparable between TGMS-TAC and systemic tacrolimus treatment. (a) Experimental scheme of the 2 treatment groups. Group 1 – 1 ml hydrogel with 7 mg tacrolimus intragraft treated group (TGMS-TAC). Group 2 daily systemic treatment with 1 mg/kg/day tacrolimus group (systemic tacrolimus), including treatment application frequencies and planned duration of the experiments. (b) Kaplan-Meier survival curve of TGMS-TAC-treated and systemic tacrolimus treated animals (n=6 / group). (c) Macroscopic grading of graft rejection over time in each of the rats (animals 1 to 6 – TGMS-TAC treated, animals 7 to 12 – systemically treated). Appearance of edema and erythema are defined as grade 1, epidermolysis and exudation as grade 2, desquamation, necrosis and mummification as grade 3. TGMS-TAC reinjection time points are indicated. Animal 1 was euthanized at postoperative day 149 after reaching grade 3 rejection. (d) Representative histological Hematoxylin and Eosin stained sections of graft skin collected at endpoint – postoperative day (POD) 280 from both treatment groups and their corresponding histopathological evaluation. Intergroup differences of the results presented are not significant as evaluated by Mann-Whitney test. Individual values are presented as dots with indication of the mean values by lines.

Figure 2 TGMS-TAC treatment does not completely prevent, but can revert rejection episodes. (a) Representative photographs of rejecting graft before (POD 204) and after (POD 234) TGMS-TAC reinjection and (b) corresponding histological Hematoxylin and Eosin stained sections of rejecting graft skin before and after TGMS-TAC reinjection.

Figure 3 Blood tacrolimus levels are lower with TGMS-TAC therapy. (a) Tacrolimus levels were measured by LC-MS/MS in blood of TGMS-TAC and systemic tacrolimus treated rats (trough levels) over time. TGMS-TAC reinjection time points are indicated by syringe symbols on the x-axis. Statistical analyses of the differences between the peaks of tacrolimus release under TGMS-TAC treatment are shown. Data are shown as individual values and means, *** $P < 0.001$, **** $P < 0.0001$ by ordinary one-way ANOVA. Individual values have been manually shifted to left or right to make each value visible. (b) Mean biweekly tacrolimus blood levels between TGMS-TAC and systemic tacrolimus treated rats are compared. Each data point represents the mean value of pooled tacrolimus measurements acquired over the course of each 2 consecutive weeks (starting from postoperative week 1 + 2, then 3 + 4 etc. until the end of the study). Statistical analyses of the differences between the 2 groups are shown. Data are presented as individual values, mean \pm SD. are indicated, ** $P < 0.01$ by Mann-Whitney test.

Figure 4 Tacrolimus levels in graft skin are higher with TGMS-TAC therapy. (a) LC-MS/MS tacrolimus measurements in graft and contralateral limb skin biopsies of TGMS-TAC-treated rats over time. TGMS-TAC reinjection time points are indicated. (b) LC-MS/MS tacrolimus measurements in graft and contralateral limb skin biopsies of systemic tacrolimus treated rats over time. Data are shown as individual values and means. Individual values have been manually shifted to left or right to make each value visible. (c) Mean tacrolimus levels in graft and contralateral limb skin of TGMS-TAC-treated or systemic tacrolimus treated animals. Each data point represents the mean value across the experimental group at each time point of tacrolimus measurements in either graft or contralateral limb skin. Statistical analyses of the differences between the 2 groups are shown. Data are shown as individual values, mean \pm SD

are indicated, $*P<0.05$, Mann-Whitney test is used for comparisons between the 2 treatment groups, Wilcoxon test is used for comparisons within a group between the 2 sites of biopsy collections.

Figure 5 Local and systemic tacrolimus treatment is associated with low donor-specific antibody (DSA) formation. (a) Levels of IgG and IgM DSA in the plasma of TGMS-TAC or systemic tacrolimus treated rats at POD 280. Donor-thymocytes have been incubated with heat-inactivated recipients' plasma isolated at POD 280, followed by staining with anti-IgG and anti-IgM antibody. DSA deposition on thymocytes have been analyzed by flow cytometry and expressed as mean fluorescence intensity (MFI). Lower threshold for positivity is defined as mean + 2x SD of the MFI measured in donor-thymocytes incubated with naïve Lewis rats' plasma. Data are shown as individual values, mean \pm SD are depicted, $*P<0.05$ by Student's t-test. (b) Representative results of immunostained graft skin and muscle cryosections with anti-IgG or anti-IgM antibody and (c) their corresponding quantification. Data are shown as individual values, mean \pm SD are depicted. Intergroup differences are not significant as evaluated by Student's t-test.

Figure 6 TGMS-TAC treatment mitigates elevation of kidney function markers and occurrence of malignancy and opportunistic infections. (a-f) Biochemical analyses of the plasma levels of (a) blood urea nitrogen, (b) creatinine, (c) cholesterol, (d) triglycerides, (e) aspartate aminotransferase, (f) alanine aminotransferase in plasma collected at POD 280 from TGMS-TAC and systemic tacrolimus treated rats. Data are shown as individual values, mean \pm SD are depicted, $*P<0.05$, $**P<0.01$, $****P<0.0001$ by one-way ANOVA. (g)

Representative histological Hematoxylin and Eosin stained section of pseudocyst infected with *Staphylococcus aureus* and *Proteus mirabilis* in a systemic tacrolimus treated rat and of (h) ipsilateral inguinal lymph node with lymphoma in another systemic tacrolimus treated rat.

Figure 7 TGMS-TAC treatment depletes T cells to a lesser extent than systemic TAC treatment. (a-f) Absolute number of T cell populations in blood of TGMS-TAC and systemic tacrolimus treatment at selected time points. The values measured in naïve animals are reported (black dots). Cells are enumerated by flow cytometry as (a) T cells (CD45⁺, CD3⁺), (b) Cytotoxic T cells (CD45⁺, CD3⁺, CD8⁺), (c) T helper cells (CD45⁺, CD3⁺, CD4⁺), (d) Treg cells (CD45⁺, CD3⁺, CD4⁺, FoxP3⁺, CD25^{high}), (e) Helios⁺ Treg cells (CD45⁺, CD3⁺, CD4⁺, FoxP3⁺, CD25^{high}, Helios⁺), (f) Helios⁻ Treg cells (CD45⁺, CD3⁺, CD4⁺, FoxP3⁺, CD25^{high}, Helios⁻). Data are shown as mean ± SD. Statistical analyses between naïve and the 2 treatment groups - one-way ANOVA. Highlighted in orange are the time points, which are significantly different between TGMS-TAC and systemic tacrolimus treatment groups. Significant differences between naïve animals and treated animals are not shown.

Figure 8 TGMS-TAC boosts multilineage hematopoietic chimerism in blood. (a-f) Donor-derived white blood cell populations in blood from TGMS-TAC and systemic tacrolimus at selected time points. The values measured in naïve animals (unspecific-staining) are reported (black dots). Donor cells are enumerated by flow cytometry as (a) all donor-derived white blood cells (CD45⁺, RT1a⁺), (b) donor-derived B cells (CD45⁺, CD3⁻, CD4⁻, SSC^{low}, RT1a⁺), (c) donor-derived T helper cells (CD45⁺, CD3⁺, CD4⁺, RT1a⁺), (d) donor-derived cytotoxic T cells (CD45⁺, CD3⁺, CD8⁺, RT1a⁺), (e) donor-derived monocytes (CD45⁺, CD3⁻, CD4⁺, RT1a⁺), (f)

donor-derived granulocytes (CD45⁺, CD3⁻, CD4⁻, SSC^{high}, RT1a⁺). Naïve Lewis rats' white blood cells are RT1a⁻. Data are shown as mean \pm SD. Statistical analyses between naïve and the 2 treatment groups - one-way ANOVA. Highlighted in orange are the time points, which are significantly different between TGMS-TAC and systemic tacrolimus treatment groups. Significant differences between naïve animals and treated animals are not shown.

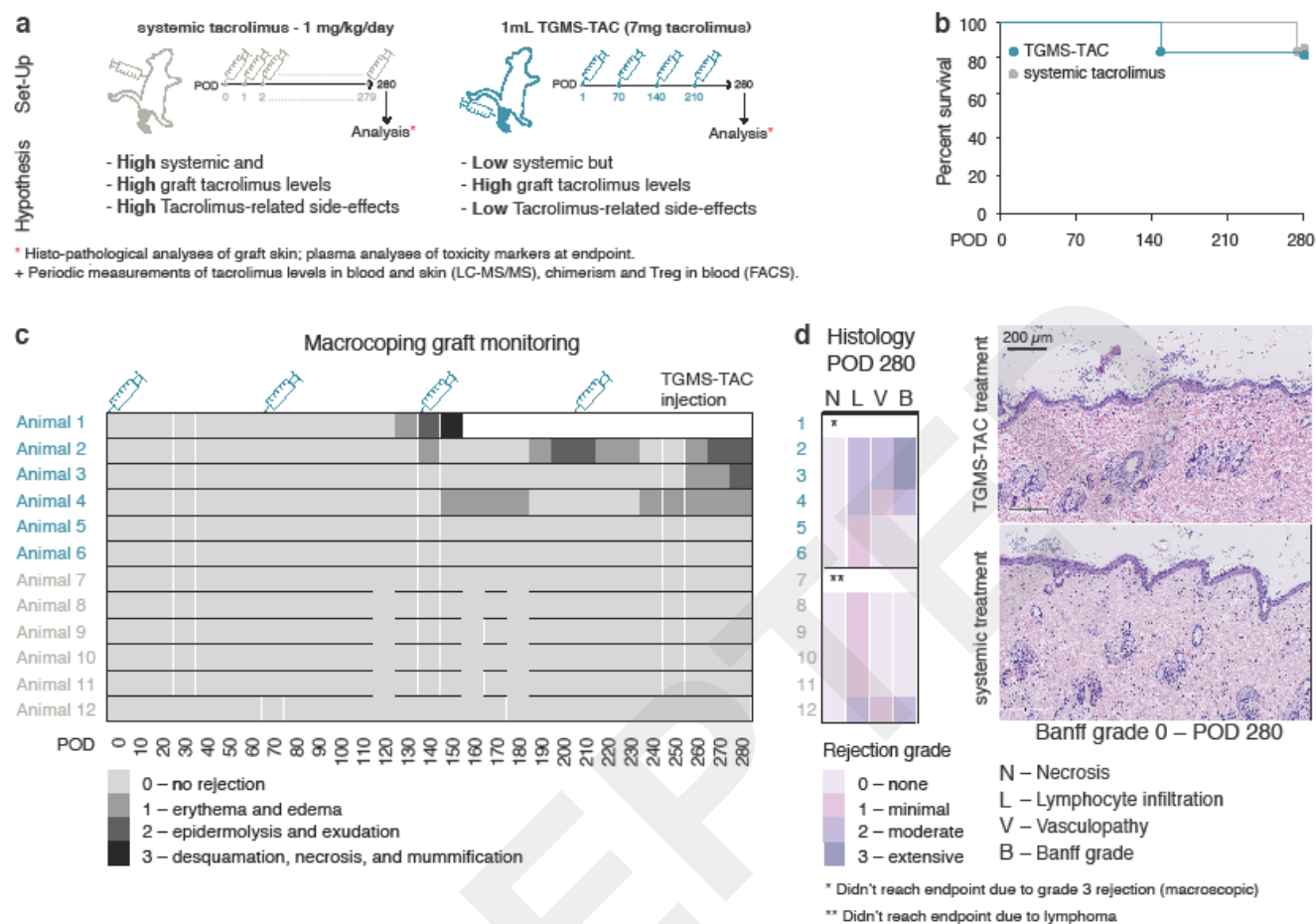


Figure 1

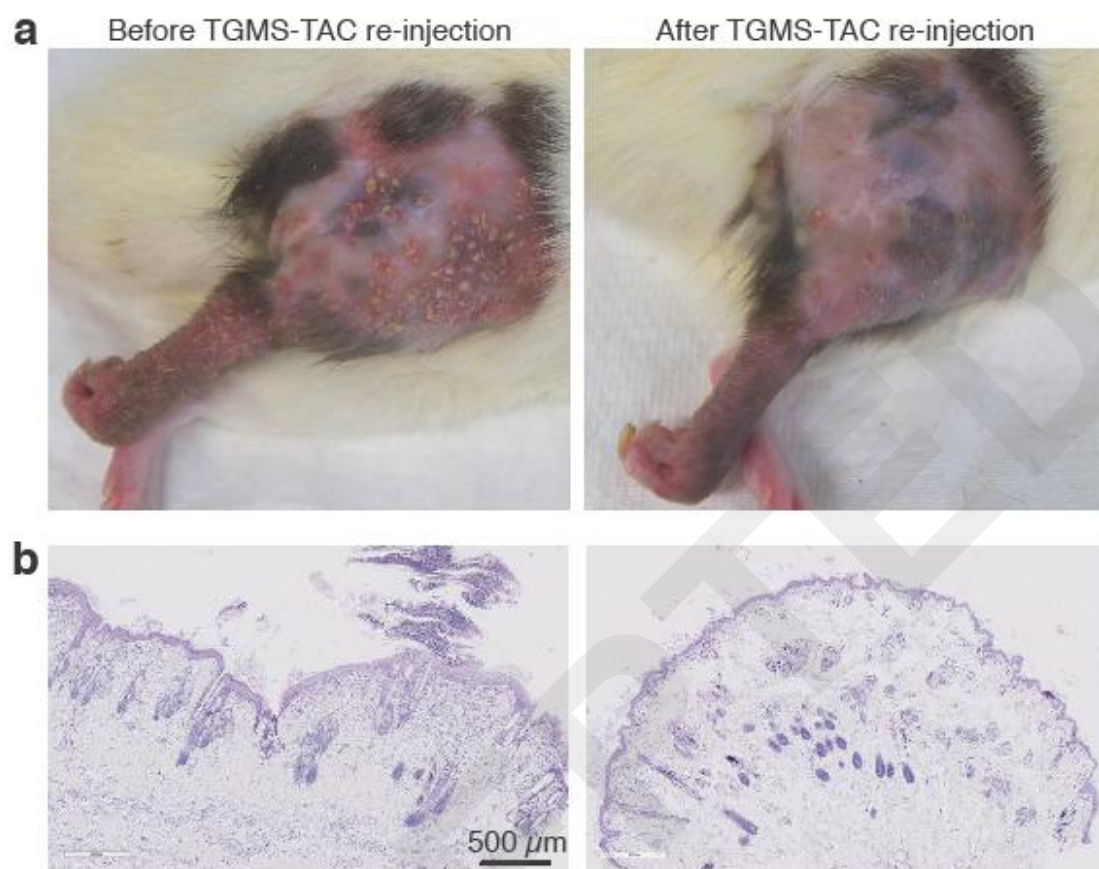


Figure 2

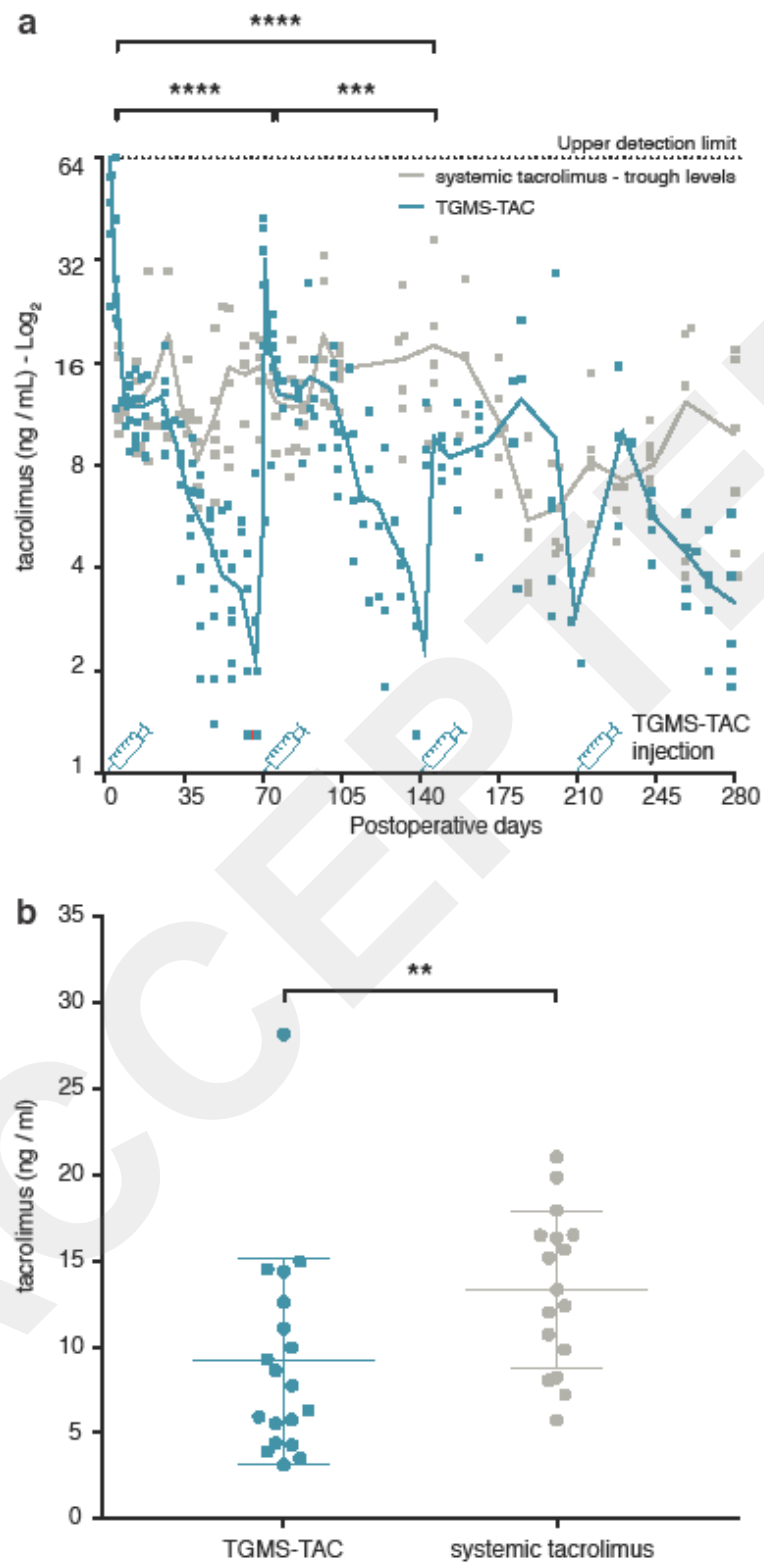


Figure 3

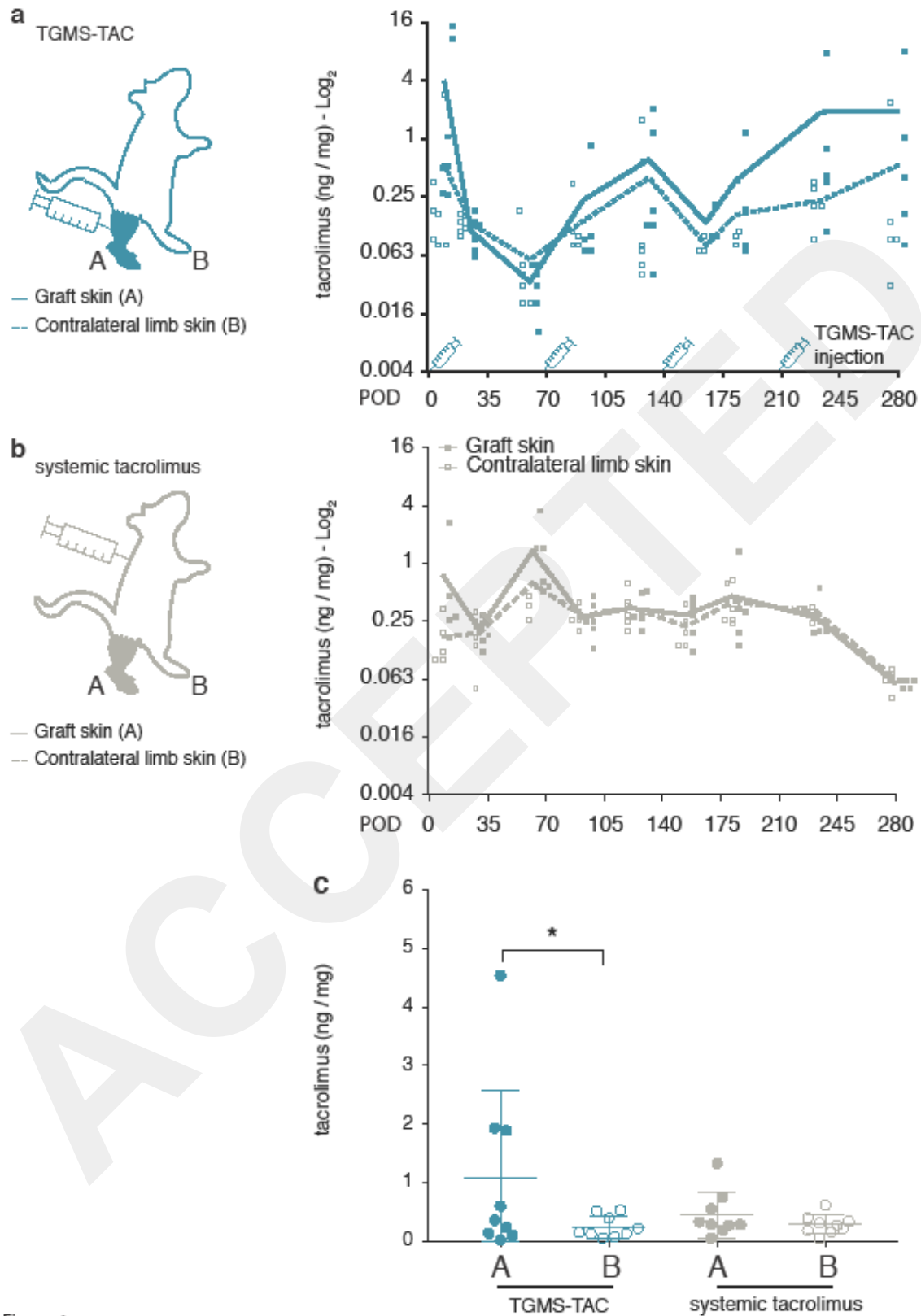


Figure 4

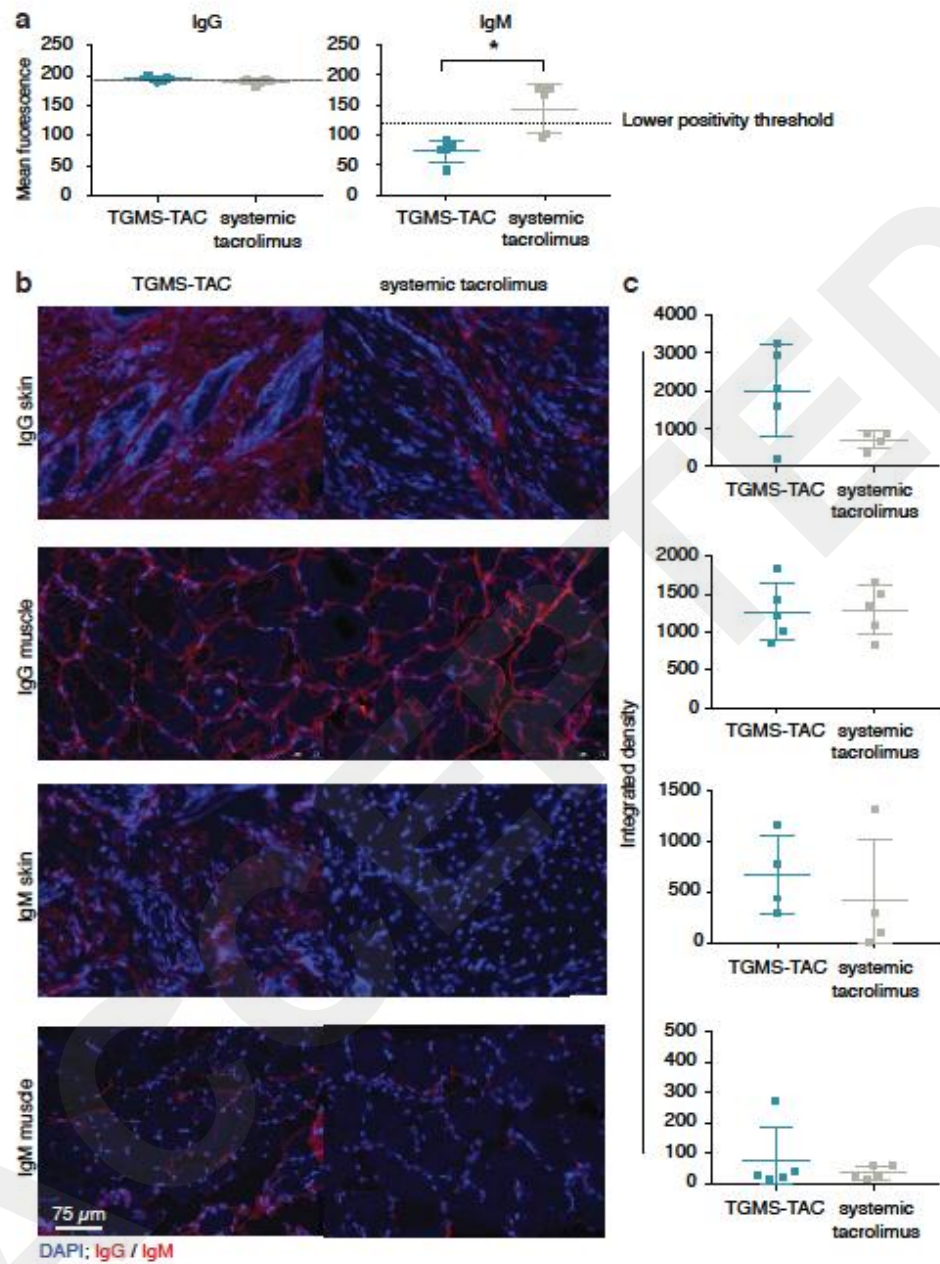


Figure 5

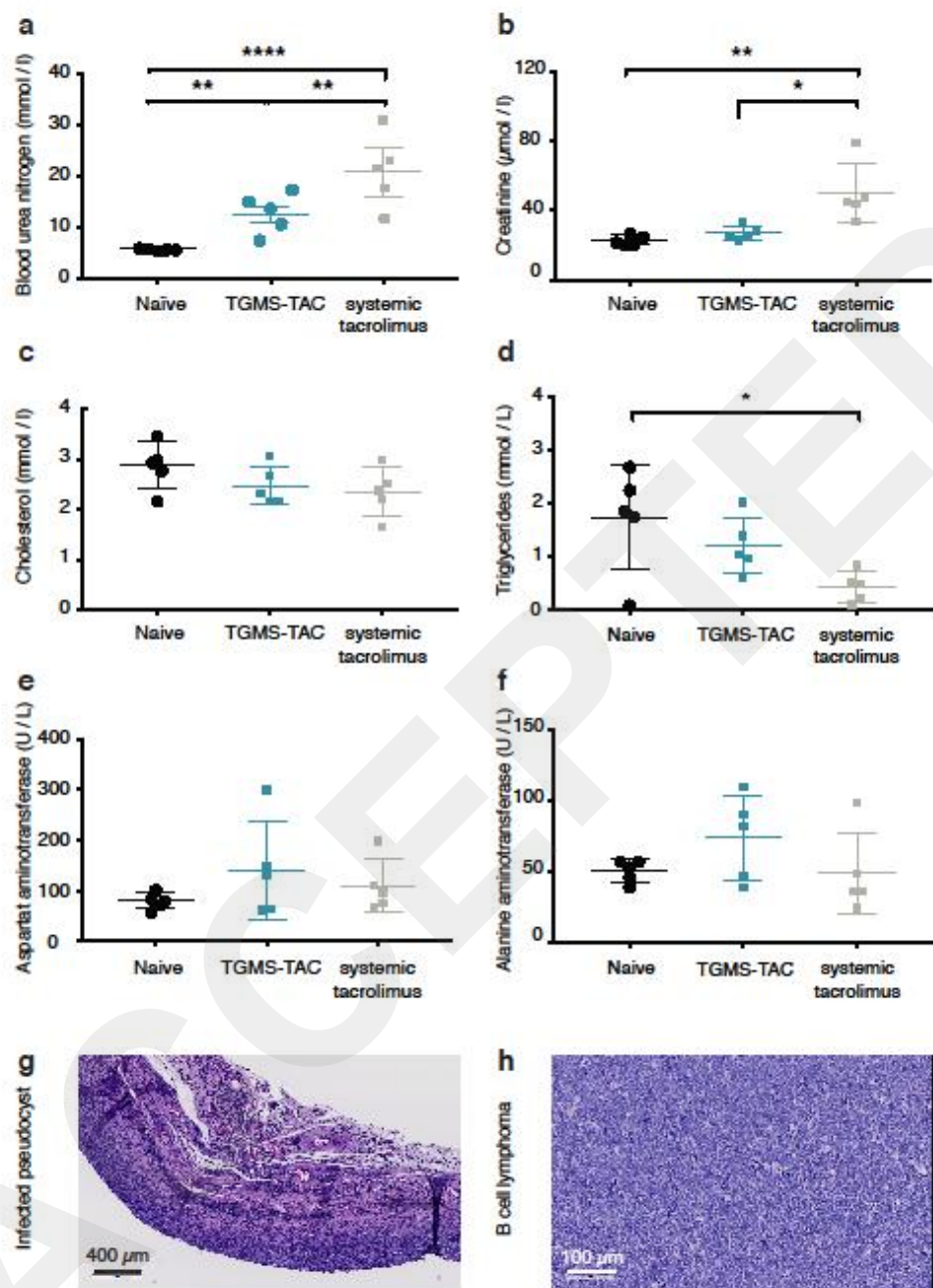


Figure 6

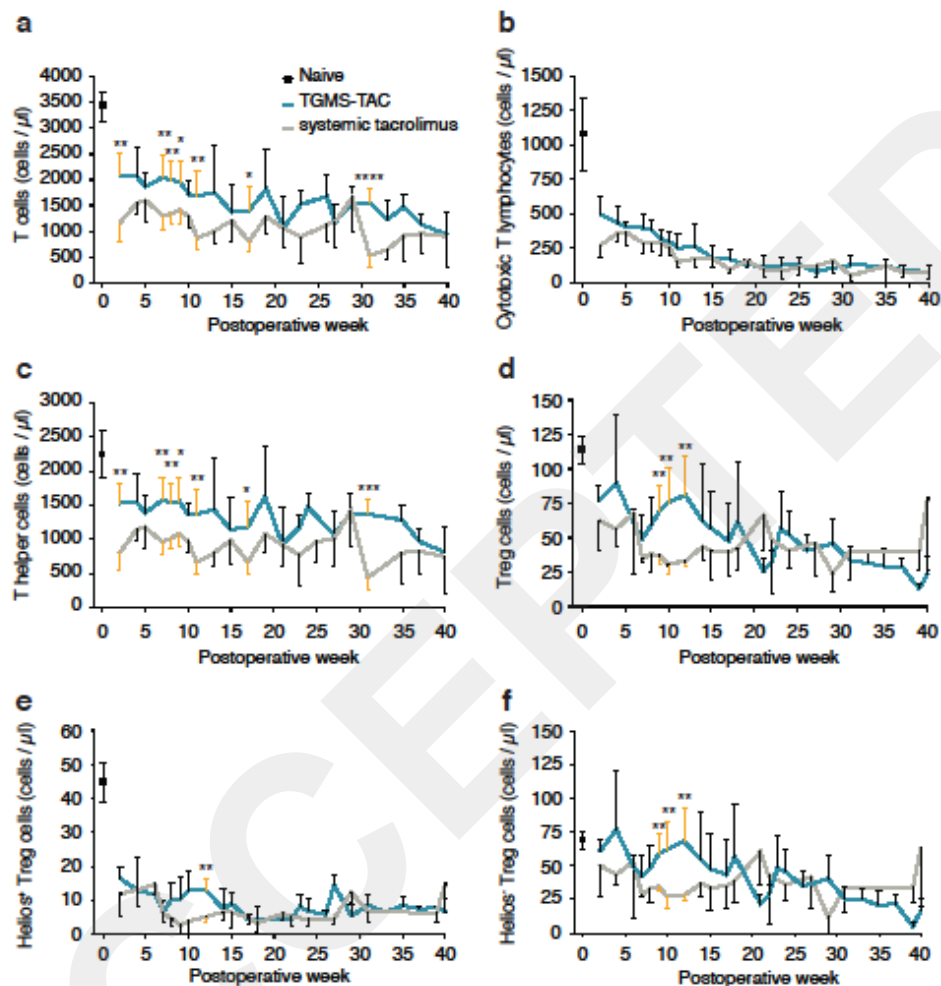


Figure 7

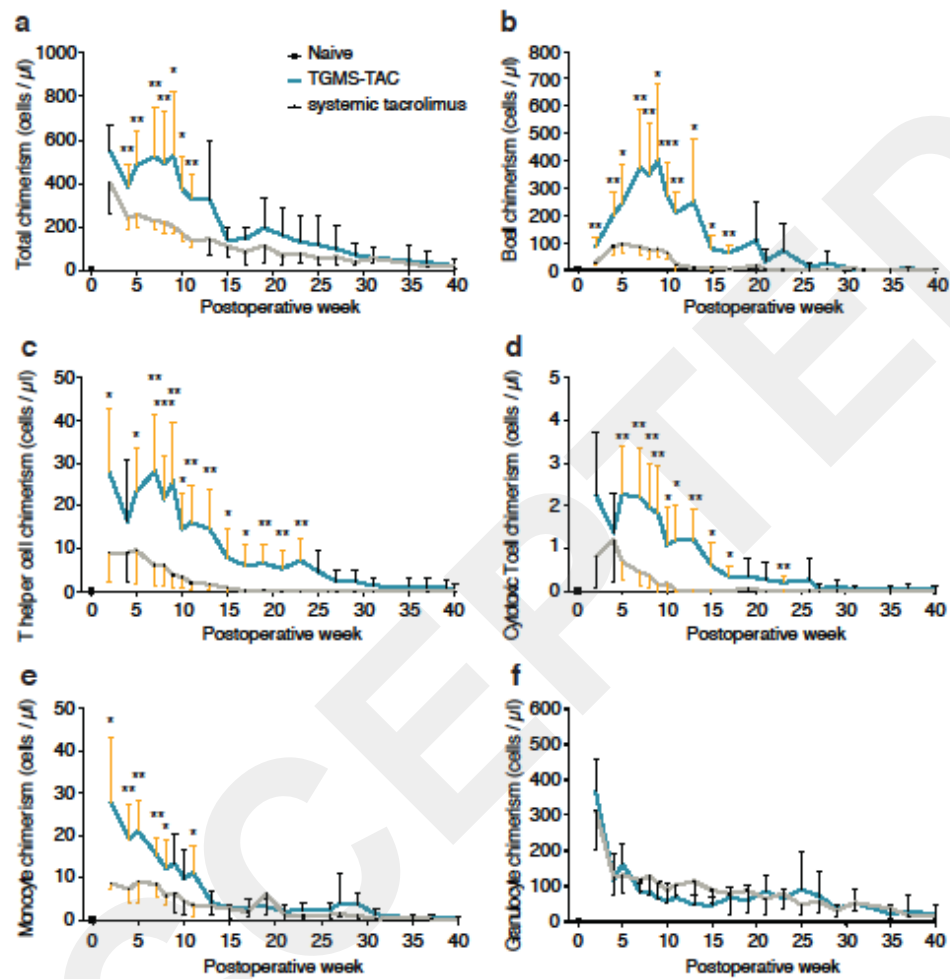


Figure 8

Table 1 Complete blood count of TGMS-TAC and systemic tacrolimus treated groups at POD 280 vs. naive age-matched animals

	Naive Lewis rats (n=9)	TGMS-TAC (n=5)	Systemic tacrolimus (n=5)	Significance
WBC x 10 ³	9.6 ± 0.9	7.4 ± 3.4	14.8 ± 15.1	ns
RBC x 10 ³	9.5 ± 0.9	10.1 ± 0.6	9.6 ± 0.3	ns
HB g/dL	15.4 ± 0.4	15.8 ± 0.7	14.2 ± 0.7	†**, ‡**
HCT %	50.4 ± 3.7	53.4 ± 3.3	49.9 ± 1.5	ns
MCV fL	53.0 ± 2.0	53.1 ± 0.9	51.9 ± 2.1	ns
MCH pg	16.3 ± 1.4	15.7 ± 0.6	14.8 ± 1.1	ns
MCHC g/dL	30.7 ± 1.6	29.6 ± 1.0	28.5 ± 1.4	†*
PLT x 10 ³	709.2 ± 82.2	584.6 ± 124.6	629.6 ± 132.3	ns
RDW_SD fL	28.5 ± 0.4	28.7 ± 0.5	28.4 ± 0.5	ns
RDW_CV%	14.7 ± 2.0	14.9 ± 1.8	14.8 ± 2.1	ns
PDW fL	11.1 ± 0.4	11.0 ± 0.7	10.0 ± 0.3	†**, ‡**
MPV fL	8.2 ± 0.2	8.2 ± 0.3	7.7 ± 0.2	†**, ‡**
WBC - white blood cells				
RBC - red blood cells				
HB - hemoglobin				
HCT - hematocrit				
MCV- mean corpuscular volume				

MCH - mean corpuscular hemoglobin

MCHC - mean corpuscular hemoglobin concentration

PLT - platelets

RDW - red blood cell distribution width

PDW - platelet distribution width

MPV - median platelet volume

† Statistical significance between Naive Lewis rats and systemic tacrolimus treated group

‡ Statistical significance between TGMS-TAC-treated group and systemic tacrolimus treated group

* $P < 0.05$

** $P < 0.01$

SDC, Materials and Methods

Animals

Male Brown Norway and Lewis rats (6-8 weeks old weighing 200 to 250 g) were purchased from Charles Rivers Breeding Laboratories, Germany. Animals were kept in specific pathogen-free conditions and care was carried out in strict accordance with the Swiss Laws in Animal Protection. All experimental protocols were approved by the Veterinary Authorities of the Canton Bern.

Drug preparation

Tacrolimus (LC Laboratories, Woburn, MA, United States), TGMS (AK Scientific, Union City, CA, United States), EDTA Hybri-Max (Sigma, St. Louis, MO, United States) and sterile water (B.Braun, Melsungen, Germany) were used for TGMS-TAC preparation as described previously¹.

Limulus amebocyte lysate test (Pyrogen 03 Plus, Lonza Group, Basel, Switzerland) was used for pyrogen detection according to manufacturer's instructions and TGMS-TAC was considered pyrogen-free if 1:10 dilution of hydrogel in sterile water resulted negative to the test.

TAC solution for systemic injections was prepared by dissolving TAC in Ethanol (absolute, Merck, Darmstadt, Germany) with addition of 1:1 Kolliphor EL (Sigma). The solution was further diluted in sterile saline 1:10 for injection.

Hind limb transplantation and monitoring

Hind limb transplantations were performed as described previously² using a 2-surgeon method.

All the operations were performed under continuous inhalation anesthesia. Isoflurane 5% (AbbVie AG, North Chicago, IL, United States) with oxygen (0.8 L/min) was used for the induction of anesthesia. Maintenance anesthesia employed 1-1.5 % Isoflurane with 0.6 L/min oxygen. All the rats were maintained at a normal body temperature using thermal pads. Surgeon 1 prepared both hind limbs of a donor Brown-Norway rat for transplantation. The hind limbs were harvested while keeping the whole inguinal fat pad with its lymph nodes in the graft and taking care not to injure the epigastric vessels that ensure its vascularization. The femoral artery and vein were prepared for anastomosis on a length of approximately 1-1.5 cm from the emergence of the epigastric vessels up to the inguinal crease. Concomitantly, surgeon 2 prepared the first Lewis recipient by removing the hind limb including the inguinal fat pad and performing a midfemoral amputation. The recipient vessels were prepared for anastomosis from the inguinal crease up to the emergence of the epigastric vessels. The first allograft was then reattached by surgeon 2 using and intramedullary osteosynthesis with a 19 G needle with blunted ends, after which preliminary fixation of the anterior muscle compartments was performed with resorbable 5-0 sutures. Venous anastomosis was performed next performed using the cuff technique with a polyimide cuff with an inner diameter of 1.19 mm (Vention Medical Inc Denver, United States). The arterial anastomosis was performed next in an end-to-end microsurgical technique using 10-0 ETHILON Nylon Suture (Ethicon Inc.,

Somerville, NJ, United States). The transplantation was completed after neurorrhaphy of the femoral nerve, sciatic nerve and approximation of the posterior compartment thigh muscles. Skin closure was performed with resorbable 5-0 Coated VICRYL (polyglactin 910) Suture (Ethicon Inc.) in a continuous fashion. Surgeon 1 performed the same procedure of transplantation of the second hind limb allograft to a second Lewis recipient after finishing graft procurement and euthanasia of the BrownNorway rat. The successfully transplanted rats were randomly assigned to the following 2 groups:

Group 1 - Systemically treated with daily subcutaneous injection in the neck fold of 1 mg/kg TAC solution (n=6); Group 2 – Treated with 1 ml TGMS-TAC loaded with 7 mg TAC subcutaneously in the graft every 70 days starting the day after the operation (n=6). In this group, 4 depots of TGMS-TAC of 250 μ L each were injected in the zones of biceps femoris, gastrocnemius, tibialis anterior, and vastus muscles, taking great care to distribute the amount of drug as evenly as possible intra and interindividually. Reinjection time point was decided upon a pilot study showing that transplanted animals (n=5) treated with a single intra- and inter-graft injection of 1 ml TGMS-TAC loaded with 7 mg TAC (POD 1) rejected their graft on POD 83.4 ± 6.7 . Reinjection time point was defined as 14 days before rejection and set to POD 70.

After transplantation, animals were inspected on a daily basis for weight loss and signs of pain or distress³. Graft survival was monitored until endpoint – POD 280 or macroscopic grade 3 rejection. Graft rejection was evaluated macroscopically as grade 0 = no rejection, 1 = erythema and edema, 2 = epidermolysis and exudation, and 3 = desquamation, necrosis, and mummification. At sacrifice skin and muscle from the graft were formalin fixed (24 h), paraffin-embedded, and sectioned (3 μ m). Hematoxylin-eosin as well as periodic acid-Schiff stained sections were graded by a pathologist blinded to the treatment regimens. Skin was graded according to Banff classification⁴, while muscle necrosis and lymphocyte infiltration were graded as grade 0 – none, 1 – minimal, 2 – moderate, and 3 – extensive.

Tacrolimus kinetics analyses

TAC in blood (systemic), in graft skin biopsies (local), and in contralateral hind limb skin biopsies was measured at selected time points.

Peripheral blood was collected from the sublingual vein in EDTA coated tubes (Sarstedt, Nümbrecht, Germany) and stored at -20° C until use. TAC concentrations in blood were assessed using the Kit MS1100 (ClinMass Complete Kit, advanced, for Immunosuppressants in Whole Blood, RECIPE Chemicals + Instruments GmbH, Munich, Germany) and quantified by LC-MS/MS.

Skin biopsies from the transplanted and contralateral limb were excised, weighed, snap frozen, and stored at - 20° C until use. The sample preparation was adapted using the MS1312 from Recipe as internal standard. TAC and internal standard were dissolved in 70 % (v/v) methanol solution. Standard spiking solution was prepared to build up a calibration curve between 0.3 and 65 ng/mL. The frozen tissues were gently thawed at room temperature. For blank matrix, samples skin samples without TAC treatment were used. A blank

matrix was prepared adding 1000 µL of precipitation solution to untreated tissue. A volume of 40 µL of internal standard solution and 960 µL of precipitation solution were added to the treated samples. All samples were then grinded with 5 stainless steel balls for 30 minutes at 25 Hz. The tubes were centrifuged 5 minutes at 4° C and 20'000 rcf. 500 µL of the tissue extract was filtered with a Mini-Uni Prep G2 vials (GE Healthcare, Chicago, USA). Chromatographic analysis was performed on an Acquity I-Class system (Waters, Milford, MA, USA) with ClinMass Complete Kits (Immunosuppressants in whole blood, advanced – on-line analysis). The autosampler temperature was set at 10 °C and the autosampler needle was washed with a strong needle wash solution of isopropanol:methanol:acetonitrile:H₂O (1:1:1:1, v/v). A solution of 20 % (v/v) methanol was used as weak needle wash. Analytes were ionized by electrospray ionization (ESI) in the positive mode and detected on a triple quadrupole mass spectrometer (Xevo TQ-S, Waters, Milford, MA, USA). The capillary and the cone voltage were set at 3 kV and 40 V, respectively. The source offset was set at 60 V, the desolvation temperature at 400° C, the desolvation gas at 1000 L/h, the cone gas at 150 L/h, the nebulizer at 7 bar and the source temperature at 150° C. The instrument was controlled via MassLynx (version 4.1, Waters). Data were acquired, integrated and processed with TargetLynx (MassLynx v4.1).

Analyses of immunosuppression-related toxicity

At sacrifice complete blood count was acquired (Sysmex KX-21N automatic hematology analyzer, Sysmex Corporation, Kobe, Hyōgo Prefecture, Japan). Kidney and liver function markers in plasma (creatinine, blood urea nitrogen and alanine aminotransferase, aspartate aminotransferase respectively) were submitted for analysis to the Center of Laboratory Medicine at the University Hospital of Bern. Kidney and liver samples were formalin fixed (24 h), paraffin-embedded, and sectioned (3 µm). Histopathological analysis (Hematoxylin and Eosin, Periodic acid– Schiff) were graded by a pathologist blinded to treatment regimens as described previously⁵. Results were compared to naïve age-matched Lewis rats.

Flow Cytometry analyses

For blood analyses, freshly drawn blood was collected in EDTA coated tubes at predefined time points for chimerism and Treg analyses. Erythrocytes were lysed with 10X RBC Lysis Buffer (Multispecies, Thermo Fisher Scientific, Waltham, MA, United States) and the cells were incubated for 15 min with Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific). After washing with 1xPBS 1%BSA, cells were incubated with the following antirat antibodies: Alexa Fluor 700 antirat CD45 antibody (BioLegend, San Diego, CA, United States), CD3-PerCP-Vio700, rat (Miltenyi Biotec, Bergisch Gladbach, Germany), APC/Cy7 antirat CD4 antibody (BioLegend), CD8b-PE-Vio770, rat (Miltenyi Biotec), and either FITC antirat CD25 antibody (BioLegend), or mouse antirat MHC Class I RT1Ac:FITC (Bio-Rad Laboratories, Hercules, CA, United States) for 15 min at 4°C. Cells were washed and permeabilized (eBioscience FoxP3 / Transcription Factor Staining Buffer Set, Thermo Fisher

Scientific) and incubated 30 min with anti-Helios-PE, human and mouse (Miltenyi Biotec) and FOXP3 monoclonal antibody (FJK-16s), eFluor 450 (Thermo Fisher Scientific), and acquired using a LSR II cytometer (BD Biosciences, San

Jose, United States) equipped with FACS Diva Software (BD Biosciences). Data were analyzed with FlowJo software (Tri-Star, Ashland, United States). Absolute cell number was estimated from the proportion of cells recorded by flow cytometry in the CD45+ gate multiplied by absolute mononuclear cell count measured using a Sysmex hematology analyzer in the same blood sample.

For skin analyses, at sacrifice graft and contralateral limb skin was minced (gentleMACS dissociator, Miltenyi Biotec, Bergisch Gladbach, Germany) and digested with Collagenase D (Roche, Basel, Switzerland) 1 mg/ml and DNase I (Sigma) 200 µg for 1h at 37° C on agitation. Resulting suspension was filtered through 40 µm cell strainers (Falcon, Corning Inc., Corning, NY, United States) and overlaid on top of Ficoll-Paque PLUS Separation Media (GE Healthcare, Little Chalfont, United Kingdom). After centrifugation the ring of cells was collected, washed, and used immediately. Cells were stained, acquired and analyzed as described above.

DSA analyses in plasma

Plasma isolated at sacrifice was complement inactivated (46° C, 30 min) and incubated (1:10 in PBS) for 15 min with donor thymocytes (1×10^5 cells) pretreated with 3% BSA and purified mouse antirat CD32 (BD Biosciences) to block unspecific Fc receptors binding. After washing, cells were incubated for 15 min with Fixable Viability Dye eFluor 506 (Thermo Fisher Scientific), washed and stained with CD3-PerCP-Vio700, rat (Miltenyi Biotec), goat F(ab')₂ anti rat IgG:FITC (Bio-Rad Laboratories) and R-Phycoerythrin AffiniPure F(ab')₂ fragment goat antirat IgM, µ chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) for 15 min at 4°C. After extensive washing, cells were acquired and analyzed as explained above. Minimum threshold of positivity for DSA in the plasma of transplant recipients was determined to be equal to the mean of naïve Lewis plasma plus 2 times its standard deviation.

Immunofluorescence analyses

Skin and muscle samples retrieved at sacrifice day were embedded in TissueTec - O.C.T. (Sakura Finetek, Alphen aan den Rijn, The Netherlands) on dry ice and sectioned (5 µm). Slides were stained with DAPI (4',6-diamidino-2-phenylindole, Boehringer Mannheim GmbH, Mannheim, Germany) and 1 of the following primary antibodies: goat antirat IgG-BIOT (Southern Biotech, Birmingham, AL, United States), goat antirat IgM-BIOT (Southern Biotech), mouse anti rat CD45RA (B Cells Only, Southern Biotech), polyclonal rabbit antihuman C3c complement (Multipurpose, Agilent, Santa Clara, California, United States), anticomplement C4c antibody (LifeSpan BioSciences, Seattle, WA, United States) or C5b-9, rat, mAb 2A1 (Hycult Biotech, Plymouth Meeting, PA, United States). The following secondary antibodies were used: Streptavidin-Cy3 from *Streptomyces avidinii* (Sigma-

Aldrich), antirabbit IgG (whole molecule), F(ab')₂ fragment–Cy3 antibody produced in sheep (Sigma-Aldrich), donkey antisheep IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific) or goat antimouse IgG (H+L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 488 (Thermo Fisher Scientific). Slides were visualized with Leica DMI4000, LAS AF Software, Wetzlar, Germany. All images were captured with identical exposure times. Quantitative analysis of fluorescence intensity (integrated density) was performed by ImageJ software (<https://imagej.nih.gov/ij/>).

Statistical analyses

Statistical analyses were performed with Prism software (GraphPad Software Inc., La Jolla, CA, United States). Statistically significant data are presented as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; and **** $P < 0.0001$. Tests are specifically indicated under each figure.

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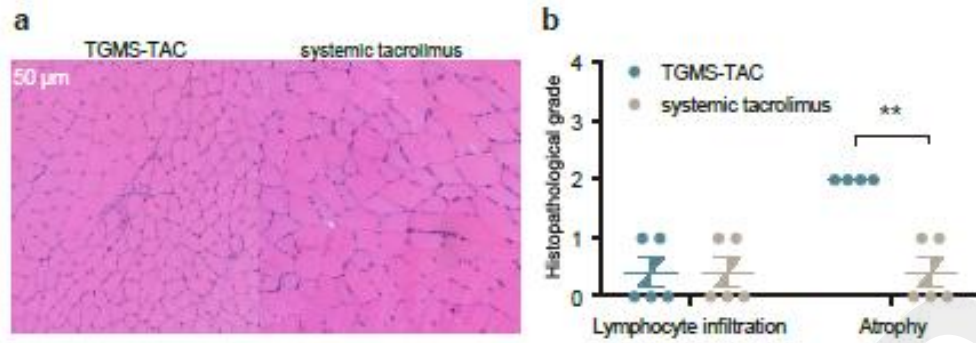


Figure S1: Atrophy and no rejection in graft muscle. (a) Representative histological hematoxylin and eosin stained sections of graft muscle collected at POD 280 from animals treated with TGMS-TAC or systemic tacrolimus and their corresponding (b) histopathological evaluation. Data are presented as individual values, mean \pm s.d are indicated, **P<0.01 by Mann-Whitney test.

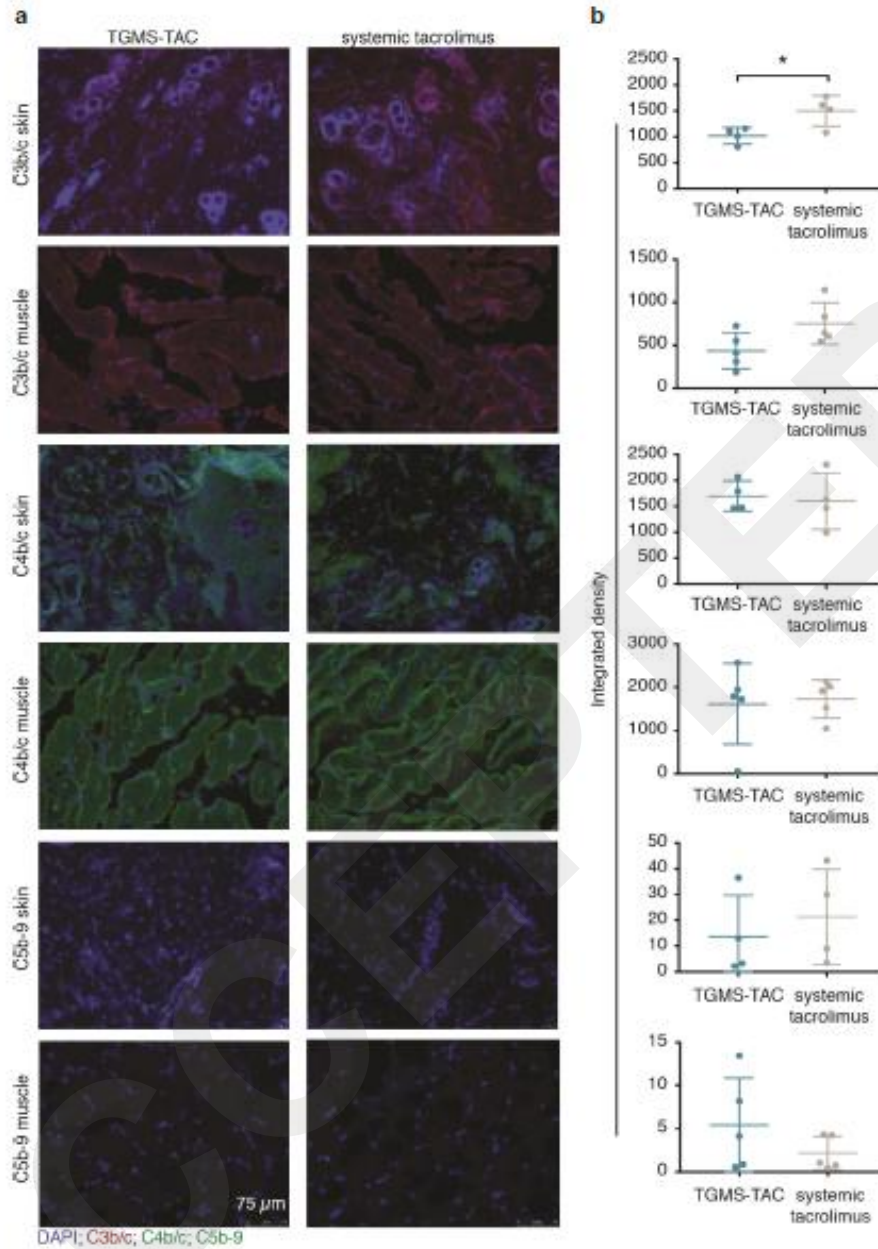


Figure S2: No complement deposition is detectable in long-term surviving grafts. C4b/c, C3b/c, C5b-9 deposition in graft skin and muscle retrieved at POD 280 from TGMS-TAC and systemic tacrolimus treated rats. (a) Representative results of immunostained cryo-sections with DAPI (nucleus), anti-IgG and anti-IgM antibodies and (b) their corresponding quantification. Data shown as individual values, mean \pm s.d are depicted. * <0.05 by Student's t-test.

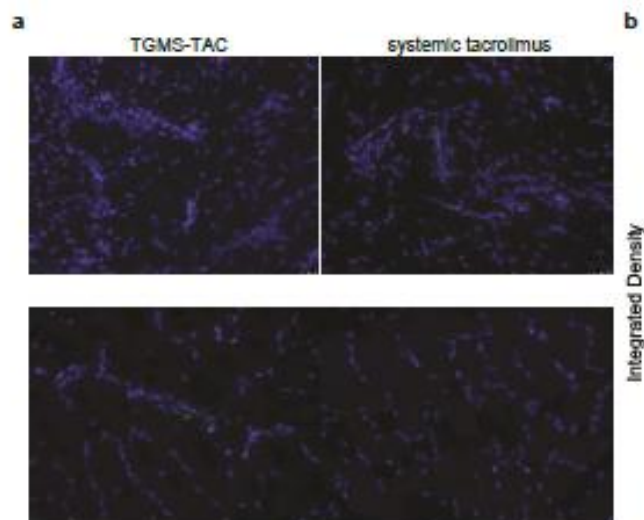


Figure S3: No B cell infiltration is detectable in long-term surviving grafts. CD45R+ cells infiltration in graft skin and muscle retrieved at POD 280 from TGMS-TAC and systemic tacrolimus treated rats. (a) Representative results of immunostained cryo-sections with DAPI (nucleus) and anti-CD45R+ antibody and (b) their corresponding quantification in graft skin and muscle. Data shown as individual values, mean \pm s.d are depicted. Statistical analysis - Student's t-test.

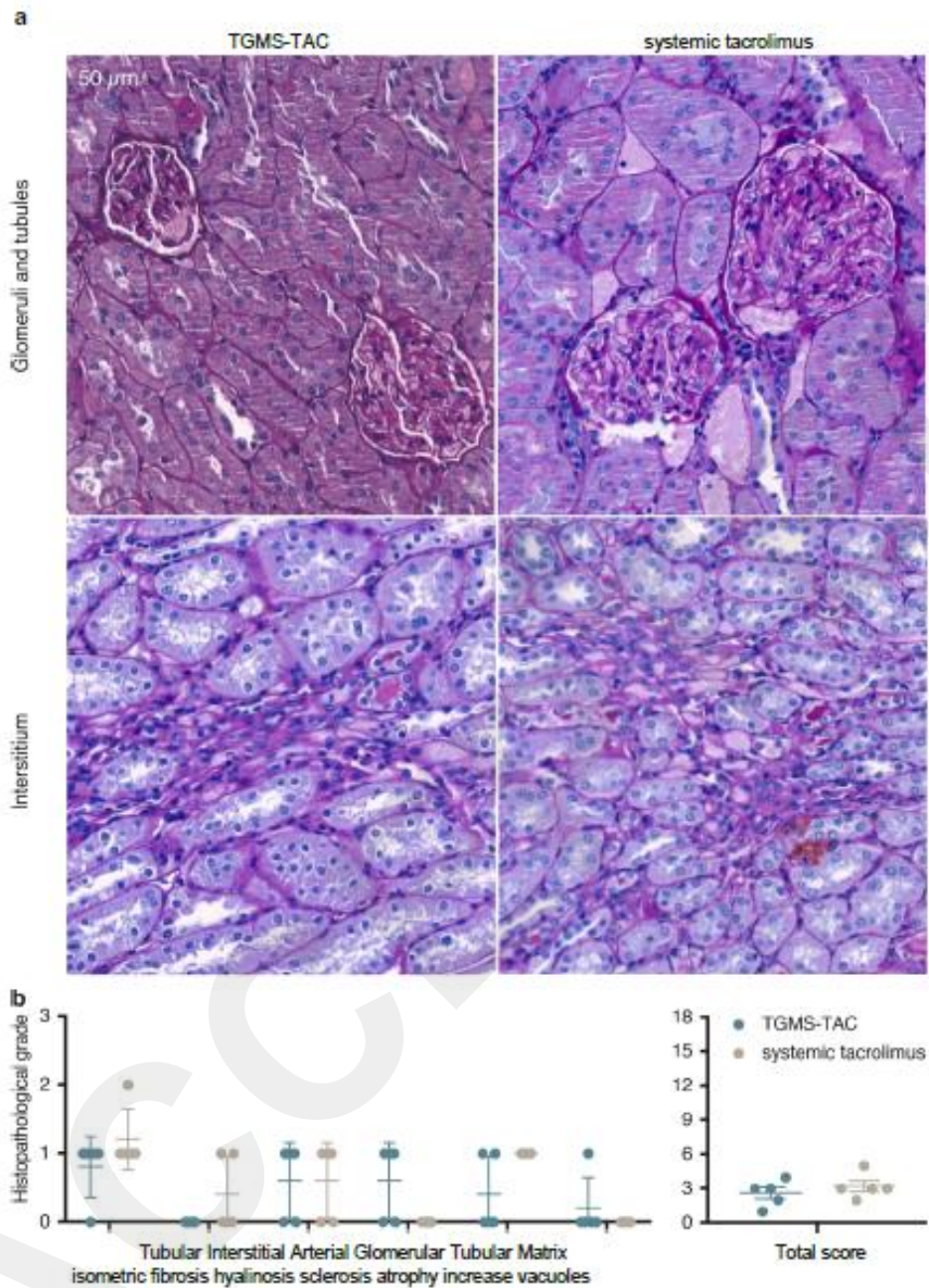


Figure S4: Minimal damage was observed in kidney of rats from both experimental groups. (a) Representative histological Periodic acid–Schiff (PAS) stained sections of kidney at POD 280 of animals treated with TGMS-TAC or systemic tacrolimus and their corresponding (b) histopathological evaluation according to the following semiquantitative pathologic scoring system for Calcineurin Inhibitor Nephrotoxicity: Tubular isometric vacuoles, Interstitial fibrosis, Glomerulosclerosis, Tubular atrophy and Mesangial matrix increase - None - Score 0, 1 to 25% - Score 1, 26 to 50% - Score 2, >50% - Score 3; Arteriolar medial hyalinosis - None - Score 0, <10% - Score 1, 11 to 30% - Score 2, >30% - Score 3. Statistical analyses of the differences between the 2 groups are shown. Data are presented as individual values, mean \pm s.d. are indicated, ** $P < 0.01$, Mann-Whitney test.

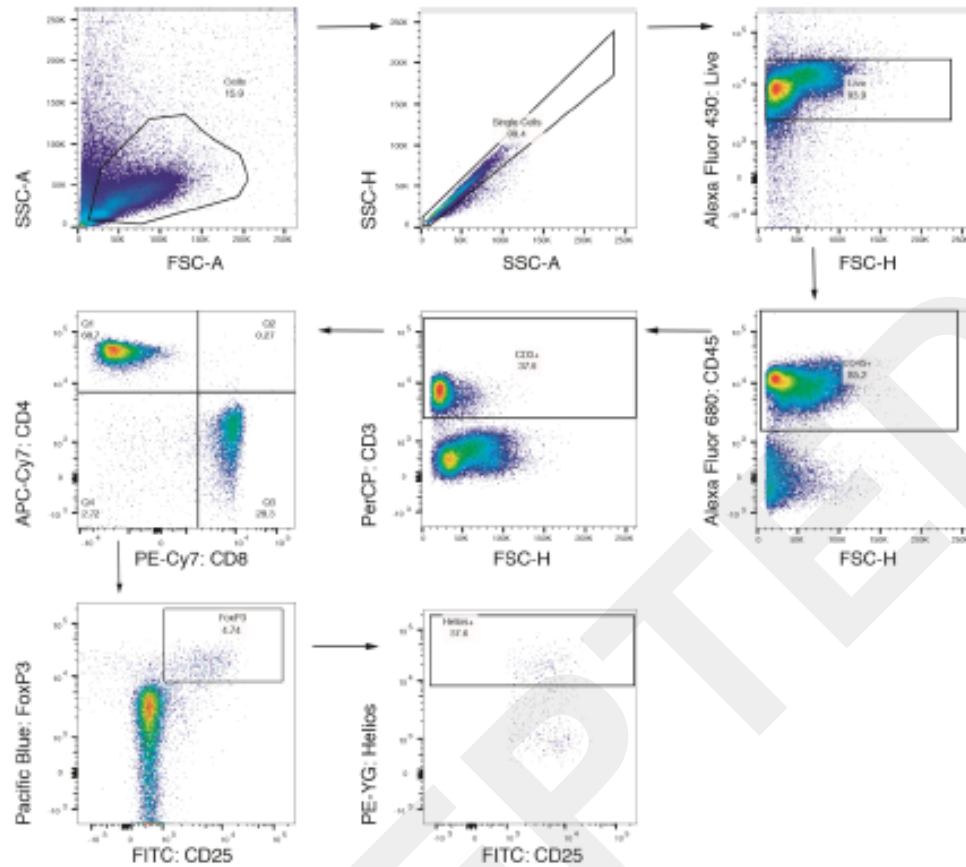


Figure S5: Gating strategy for enumeration of Treg cells in the peripheral blood. After identification of cells by their physical parameters (ie, forward and side scatter), single, viable (dim Viability Dye expression) CD45+ cells have been identified and gated. In the CD45+ gate, CD3+ cells have been selected and CD4+ population within them have been further investigated. Of those, FoxP3+, CD25high cells have been defined as Treg cells. Subsequently, Helios+ and Helios- Treg have been distinguished. All populations were expressed as frequency of CD45+ cells and the absolute cell number was determined using the complete blood cell count of the same blood sample. For skin analyses, due to inability to determine absolute white blood cell count, percent of parent population have been used to appreciate relative abundance of each population.

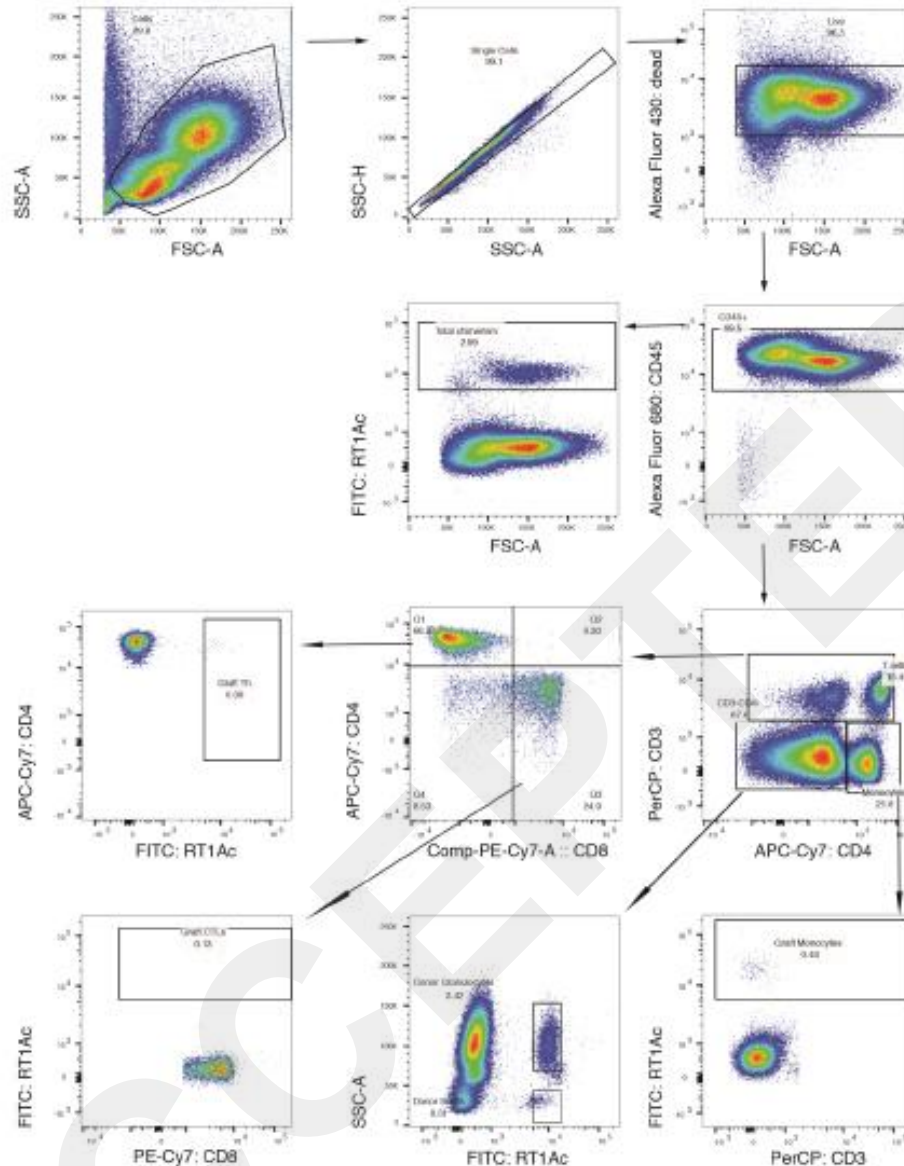


Figure S6: Gating strategy for the analysis of chimerism in the peripheral blood of hind limb recipients. After identification of cell by their physical parameters (ie, forward and side scatter), single, viable (dim Viability Dye expression) CD45+ cells have been identified and gated. In this gate the number of RT1Ac+ cells have been defined as "total chimerism" (ie, all donor-derived white blood cells). CD45+ cells have been further separated by their expression of CD3 and CD4. CD3+ cells have been defined as T cells and further separated to CD4+ T helper cells and CD8+ cytotoxic T cells. CD3-CD4+ cells have been defined as monocytes. From the CD3-, CD4- cells, SSChigh have been defined as granulocytes and SSChigh cells as B cells. In each of those populations RT1Ac+ cells have been defined as their donor-derived counterparts. For blood analyses, complete blood cell count has been used to determine the absolute white blood cells count, corresponding to the parent CD45+ population. All populations were expressed as frequency of CD45+ cells and the absolute cell number was determined using the complete blood cell of the same blood sample. For skin analyses, due to inability to determine absolute white blood cell count, percent of parent population have been used to appreciate relative abundance of each population.

a**b****c****d****e****f**

Figure S7: No significant changes in donor cell populations have been detected in graft skin. At sacrifice (POD 280) skin from both transplanted and contralateral native limb have been collected, minced and digested. Cells have been extracted and submitted to flow cytometric analyses, using the same antibodies and protocol from Figure 7 and 8. Total amount of T cells (a), Treg cells (b), Helios+ Treg cells (c) and Helios- Treg cells (d), as well as total donor-derived white blood cells (e) and T cells (f) have been analyzed according to the gating strategies described in supplementary figures 5 and 6. Populations have been presented as percent of their parent populations. Intragroup comparisons between graft and contralateral limb skin have been analyzed by paired Student's t-test. Intergroup comparisons between TGMS-TAC and systemic tacrolimus treated groups have been analyzed by unpaired Student's t-test.

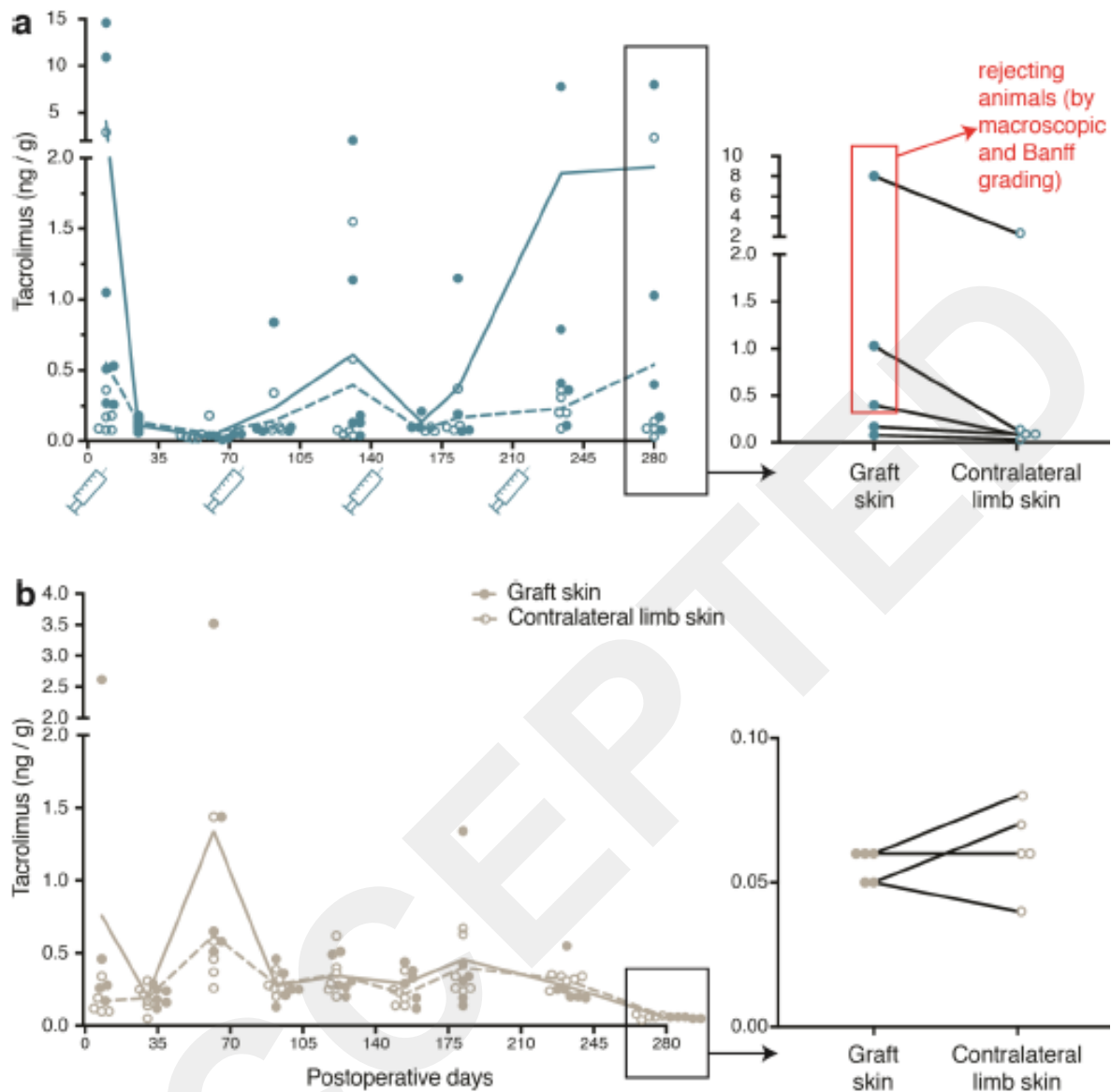


Figure S8: Trend towards higher tacrolimus levels in skin of transplanted limbs versus contralateral native limbs in rejecting TGMS-TAC treated animals. Tacrolimus measurements in graft and contralateral limb skin biopsies of (a) TGMS-TAC-treated and (b) systemic tacrolimus treated rats over time from Figure 4, with linear Y-axis. Zoomed in both panels are values from postoperative day 280 (endpoint). (a, zoomed-in panel) Tacrolimus levels in TGMS-TAC treated hind limbs are not significantly higher than in contralateral native limbs. However there appears to be a trend towards higher TAC skin levels in rejecting animals. (b, zoomed-in panel) No difference between TAC levels in transplanted and native limbs in systemically treated animals. TGMS-TAC reinjection time points are indicated. Data are shown as individual values and means. Individual values, when overlapping, have been manually shifted apart to left or right to increase visibility of each value.